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cis-Canthaxanthins

UNUSUAL CAROTENOIDS IN THE EGGS AND THE REPRODUCTIVE SYSTEM OF FEMALE BRINE SHRIMP *ARTEMIA**

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Hans J. C. F. Nelis,[a,b] Patrick Lavens,[c,d] Luc Moens,[b,e] Patrick Sorgeloos,[b,c] Jozef A. Jonckheere,[a,b] Godelieve R. Criel,[f] and André P. De Leenheer[a,g]

From the [a]Department of Medical Biochemistry, State University of Ghent, Harelbekestraat 72, B-9000 Gent, the [c]Artemia Reference Center, State University of Ghent, J. Plateaustraat 22, B-9000 Gent, the [e]Department of Cell Biology, University of Antwerp, Universiteitsplein 1, B-2610 Wilrijk, and the [f]Department of Anatomy, State University of Ghent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium

The significance of carotenoid accumulation in crustacean eggs remains obscure, particularly because neither eggs nor female animals have been found to display specific pigment patterns in relation to reproduction. We report here the first example of carotenoids found exclusively in the ovaries, the eggs, and the hemolymph, but not in the carcass of a female, reproductively active crustacean, *i.e.* the brine shrimp *Artemia*. These pigments are virtually absent in males and in immature animals and disappear very rapidly in growing nauplii following hatching of encysted embryos. Within the cysts, they are preferably localized in the yolk platelets. We have identified them as mono-*cis*-canthaxanthins on the basis of their mass and absorption spectra and by comparison with synthetic components. Carotenoids with the unusual *cis*-configuration have never been isolated from animals, nor are there reports on the occurrence of carotenoid pigments at specific sites. Our findings may thus provide a clue to a precise function for carotenoids in *Artemia* and, possibly, related Crustacea.

The function of non-provitamin A carotenoids in aquatic organisms has not been elucidated (1–3). As suggested by Cheesman *et al.* (1), carotenoids may act as prosthetic groups controlling the configuration and the stability of certain proteins. These carotenoproteins supposedly provide protective coloration or, alternatively, might participate in photochemical processes, electron transport, or enzymatic reactions (1). In view of their abundance in crustacean eggs, a possible role in embryonic development has been suggested (1), although conflicting evidence exists (4, 5). Carotenoids are indeed mobilized in the gonads at the onset of reproduction (4), and cleavage of egg carotenoproteins occurs at certain stages of development (1). However, attempts to demonstrate specific carotenoids in eggs and reproductive organs or to show qualitative differences in pigment patterns between female and male animals have failed so far (4).

While studying the carotenoid profile of the brine shrimp *Artemia*, we observed that encysted embryos of this organism contain high amounts of carotenoids with the unusual *cis*-configuration. In adults, these substances were exclusively present in the ovaries, the eggs, and the hemolymph of reproductively active females, but not in their carcass, while also being virtually absent in males. The present report is concerned with the identification of this new class of natural pigments, their distribution *in vivo*, and their fate during the *Artemia* life cycle.

EXPERIMENTAL PROCEDURES

Materials—All-*trans*-canthaxanthin was a gift from Hoffmann-LaRoche. All reagents were chemically pure and redistilled prior to use. *Artemia* cysts, nauplii, and adults came from the Artemia Reference Center (State University of Ghent, Gent, Belgium).

Methods—Liquid chromatography was carried out on a Varian 5020 chromatograph equipped with a Valco loop injector and a Varichrom variable wavelength detector, set at 470 nm. The first system consisted of a 5-μm Zorbax ODS reversed phase column (25 × 0.46 cm), eluted with a mixture of acetonitrile:methanol:dichloromethane (5:3:2), at a flow rate of 1.0 ml/min, whereas the second system used a 5-μm ROSIL silica column (20 × 0.46 cm), eluted with dichloromethane:isopropyl alcohol (99.5:0.5), at 1.0 ml/min.

Absorption spectra were recorded on line with the liquid chromatograph using an HP 1040 photodiode array detector, equipped with an HP 85 computing system, an HP 82901 M flexible disc drive, and an HP 7470A graphics plotter. Mass spectra were determined on an HP 5985 mass spectrometer via a direct insertion probe. Both electron impact (70 eV) and chemical ionization (reagent gas methane) conditions were used.

Synthetic *cis*-canthaxanthins were prepared by refluxing a solution of all-*trans*-canthaxanthin in benzene (300 mg in 150 ml) for 90 min (6). The mixture was subsequently evaporated to dryness under vacuum, and the residue was redissolved in 10 ml of dichloromethane. Small aliquots were appropriately diluted and injected on the reversed phase liquid chromatographic column to determine the *cis*/*trans* ratio. For preparative isolation, 1-ml aliquots were chromatographed on a silica column (50 × 0.9 cm; ROSIL, 8 μm) eluted with dichloromethane:isopropyl alcohol (99.5:0.5), and peaks corresponding to the individual *cis*-isomers were collected. The latter were identified on the basis of their absorption spectra. Collected fractions were concentrated under vacuum and subjected to mass spectrometric analysis. Alternatively, a saturated solution of all-*trans*-canthaxanthin in acetonitrile, with or without formic acid (4%), was refluxed, and aliquots were injected on the reversed phase column. The experimental *cis*/*trans* ratio was determined from peak height measurements.

Whole *Artemia* cysts (10 mg) were homogenized in 3 ml of ice-cold acetonitrile:methanol:dichloromethane:formic acid (46:30:20:4) containing 0.05% of butylhydroxytoluene in a Potter-Elvehjem homogenizer. After centrifugation, a 50-μl aliquot of the supernatant was

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[g] To whom correspondence should be addressed.

directly injected on the reversed phase column. All manipulations were carried out in dark brown glassware in subdued light at 0 °C and within 4 min. After water addition, carotenoids were extracted with *n*-pentane. The organic layer was evaporated to dryness under nitrogen at room temperature, and the residue was reconstituted with dichloromethane. A 50-μl aliquot was subsequently injected on the silica column. For preparative isolation of the natural pigments, 25 g of cysts were homogenized in 150 ml of a pH 7.6 buffer containing 30 mM Tris, 70 mM potassium chloride, 9 mM magnesium chloride, 150 mM sucrose, and 1 mM β-mercaptoethanol. The mixture was centrifuged at 5600 × *g* (3000 rpm) for 30 min, and the pellet was homogenized in 30 ml of solvent as described above. Carotenoids were extracted with 50 ml of dichloromethane. After drying over sodium sulfate, the organic layer was concentrated to 1 ml and injected on the semipreparative column. Upon elution, the major *cis*-canthaxanthin was trapped, the fraction was concentrated under vacuum, and an aliquot was introduced in the mass spectrometer.

Subcellular fractions were prepared by homogenization of 10 g of Reference *Artemia* cysts (7) in 40 ml of the above pH 7.6 buffer with the aid of a mortar. Following differential centrifugation at 5,600 × *g* (3,000 rpm) (10 min), 15,000 × *g* (8,000 rpm) (10 min), and 30,000 × *g* (16,000 rpm) (10 min), four fractions were obtained. The first fraction contained mainly the yolk platelets, while the second was enriched in mitochondria. On top of the post-mitochondrial supernatant (fraction 3), a flotating lipid layer (fraction 4) was present. Yolk platelets were disrupted by continuous stirring at 0 °C of a suspension in buffered sodium chloride (2 mol/liter, pH 9) as described by de Chaffoy de Courcelles and Kondo (23). The latter authors thoroughly characterized the lipovitellin, which is the principal constituent of the soluble fraction obtained after lysis. The insoluble material was found to be enriched in membranes. A 100–200-μl aliquot of each fraction was analyzed as described for whole cysts, except that the homogenization solvent contained 2% formic acid instead of 4%.

Adult animals (5–20) or nauplii (a few milligrams) were homogenized in 1–3 ml of solvent (see above), and the extracts were analyzed as described for whole cysts.

Organs and body sections of 10 reproductively active female adults were removed and immediately homogenized in 0.5–1.0 ml of solvent (containing 2% formic acid). After centrifugation, a 50-μl aliquot of the supernatant was injected. The hemolymph of 10 animals was mixed with 0.5 ml of homogenization solvent and treated as described above.

RESULTS

Demonstration of Unknown Carotenoids in Artemia—Reversed phase chromatography of extracts of dormant (dehydrated) *Artemia* cysts revealed the presence of an unknown carotenoid, eluting very close to all-*trans*-canthaxanthin (Fig. 1*A*), reportedly the major pigment in *Artemia* (8–12). This unidentified peak proved composite upon rechromatography on silica and could be further differentiated in three individual components (Fig. 1*B*). Newly hatched nauplii displayed a similar two-peak pattern in the reversed phase system except that the peak height ratio between the unknown and all-*trans*-canthaxanthin was considerably lower. The unknown pigment was also found in reproductively active females (Fig. 2*A*) but not in males (Fig. 2*B*).

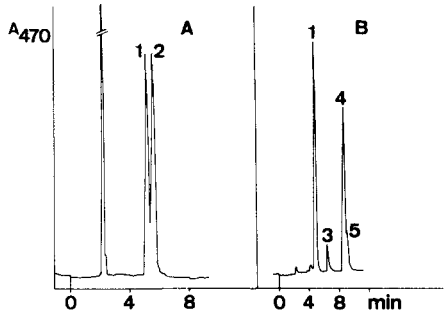

FIG. 1. **Carotenoid patterns in *Artemia* cysts.** *A*, chromatogram of an extract of Reference *Artemia* cysts obtained on a 5-μm Zorbax ODS column (25 × 0.46 cm). Eluent was acetonitrile:methanol:dichloromethane (5:3:2, v/v). Flow rate, 1.0 ml/min; detection, 470 nm. *B*, chromatogram of an extract of Reference *Artemia* cysts obtained on a 5-μm ROSIL column (20 × 0.46 cm). Eluent was dichloromethane:isopropyl alcohol (99.5:0.5, v/v). Flow rate, 1.0 ml/min; detection, 470 nm. *Peak 1*, all-*trans*-canthaxanthin; *peak 2* (composite), *cis*-canthaxanthins, further differentiated in *peaks 3–5* (*peak 5*, shoulder peak) on the silica column (*B*).

Identification of the Unknown Pigments—Canthaxanthin stereomutation mixtures yielded a similar two-peak profile in the reversed phase system as *Artemia* extracts, whereas complex, multipeak chromatograms were obtained on silica. All unknown peaks in the *Artemia* extracts had their counterpart in the stereomutation mixture. The major constituent of both the natural (Fig. 1*B*, *peak 4*) and the synthetic mixture was preparatively isolated for mass spectrometric analysis.

Their electron impact mass spectra are depicted in Figs. 3 and 4, respectively. In both spectra, fragmentation indicative for polyene structures is present in the lower mass region (13). An intense molecular ion ($M^{+\cdot}$) shows up at *m/z* 564, corresponding to the molecular weight of canthaxanthin. Typical ions were observed at *m/z* 472 ($M^{+\cdot}$ − 92) and 458 ($M^{+\cdot}$ − 106). The intensities ratio between these fragments was found to be 2.6, which is a characteristic identity criterion for canthaxanthin (13). Minor differences in peak intensities (*e.g.* peaks at *m/z* 105 and 109) between both spectra or the occurrence of "extra" peaks in either of the two should be

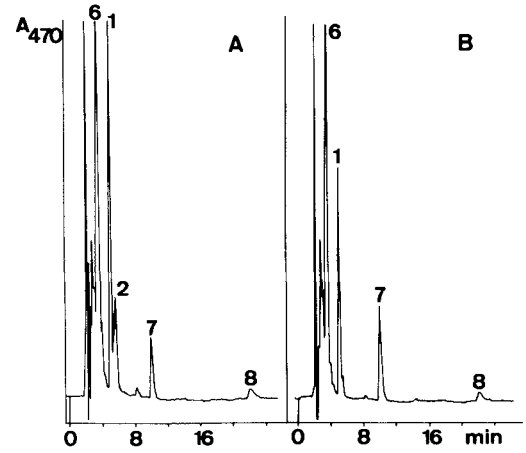

FIG. 2. **Carotenoid pattern of *Spirulina*-fed *Artemia* female (*A*) and male (*B*) adults (Macau strain, batch no. 870191).** Conditions were as described in the legend to Fig. 1*A*. *Peak 1*, all-*trans*-canthaxanthin; *peak 2*, *cis*-canthaxanthins; *peak 6*, unidentified; *peak 7*, echinenone; *peak 8*, β-carotene.

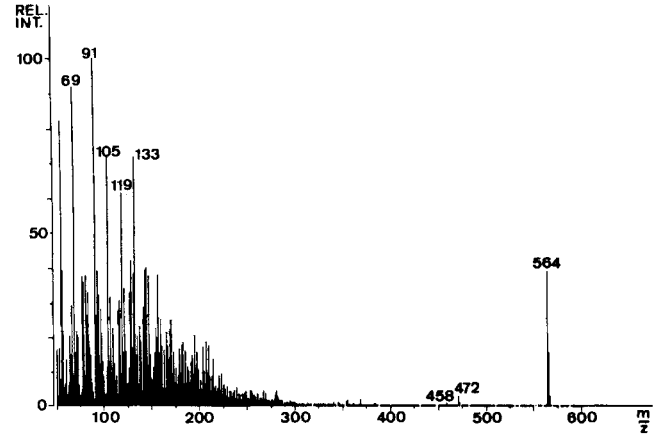

FIG. 3. **Electron impact spectrum (70 eV) of a natural *cis*-canthaxanthin (Fig. 1*B*, *peak 4*).**

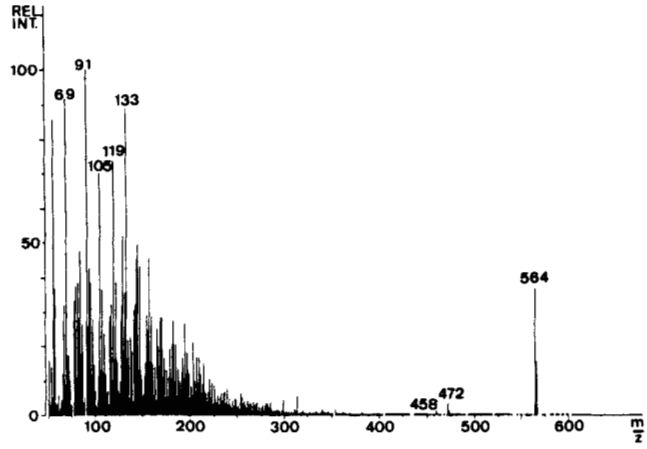

FIG. 4. **Electron impact spectrum (70 eV) of a synthetic *cis*-canthaxanthin (equivalent of *peak 4*, Fig. 1*B*).**

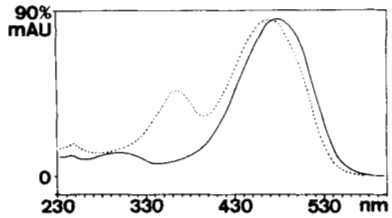

FIG. 5. **Absorption spectra of the major natural *cis*-canthaxanthin (Fig. 1*B*, *peak 4*) (*dashed line*) and of all-*trans*-canthaxanthin (*full line*).** Both spectra were recorded on line with the reversed phase system.

attributed to the presence of impurities. The limited amounts of material available indeed precluded further purification, *e.g.* by crystallization of the fractions trapped from the semi-preparative column. Furthermore, mass spectral analysis was not preceded by an on-line chromatographic step but carried out after direct inlet of the samples.

Additional evidence for the identity was obtained from the mass spectra under chemical ionization conditions (reagent gas methane). An intense quasi-molecular ion at m/z 565 (MH^+) as well as typical adduct ions at m/z 581 ($M \cdot CH_5^+$) and m/z 593 ($M \cdot C_2H_5^+$) were observed. These data led us to identify the major unknown carotenoid as canthaxanthin. Since the component is physicochemically different from all-*trans*-canthaxanthin (different chromatographic properties in two systems), it has to be a geometrical isomer. The mono-*cis*-configuration was assigned to this compound on the basis of its highly characteristic absorption spectrum (Fig. 5). The 8 nm hypsochromic shift of the long wavelength maximum (λ_{max} = 468 nm) with reference to all-*trans*-canthaxanthin (λ_{max} = 476 nm) as well as the appearance of the intense "*cis*-peak" at 363 nm, absent in the spectrum of all-*trans*-canthaxanthin, are consistent with a *cis*-carotenoid structure (6, 14–17). The two minor chromatographic peaks 3 and 5 (Fig. 1*B*) yielded similar spectra except that fraction 3 gave only a shoulder peak at 363 nm. At this point, their identification as *cis*-canthaxanthins is tentative.

Analytical Results—Table IA shows the results of the analysis of whole cysts of 12 geographical strains of *Artemia*. Both *cis*- and *trans*-canthaxanthins were always present but in variable relative proportions, the lower and upper limits (expressed as peak height ratios) being 0.52 and 1.21, respectively. Within the cysts, *cis*-canthaxanthins are protein-bound and preferably localized in the yolk platelets (Table IB), whereas all-*trans*-canthaxanthin predominates in the fraction enriched in mitochondria, the post-mitochondrial supernatant and the flotating lipids. Following lysis of yolk platelets, *cis*-canthaxanthins were found to be mainly deposited in the insoluble part, enriched in membranes, and, to a much lesser extent, in the soluble fraction containing the lipovitellin.

During embryonic development in rehydrated cysts, the *cis*/

TABLE I

Relative distribution of cis- and trans-canthaxanthin (expressed as peak height ratios) in Artemia (A–D), stereomutation mixtures (E), and heated extracts (F)

Peak height ratios were calculated from the respective peak heights obtained at a detection wavelength of 470 nm with 16 nm bandwidth. The relative proportions of the isomers can be calculated by multiplying these values by 1.5, this factor being the ratio between the molar absorption coefficients of the *cis*- and *trans*-isomers at this wavelength (6). Thus, a peak height ratio of 1.21 represents a sample containing about 65% *cis*- and 35% *trans*-canthaxanthin.

A. Whole cysts of different geographical origin

Strain	Ratio
Macau, Brazil, batch 870191	1.21
San Pablo Bay, CA, batch 1628	1.15
Reference *Artemia* cysts (7)	1.11
San Francisco Bay, CA, batch 2149	0.91
San Francisco Bay, CA, batch 2016	0.87
Tumbes, Peru, harvest 1982	0.86
Great Salt Lake, UT, batch 18.02	0.86
Shark Bay, Australia, batch 2998	0.85
Macau, Brazil, harvest 1978	0.84
Lavalduc, France, harvest 1980	0.83
Russian strain (Siberia), harvest 1982	0.75
Tientsin 253	0.52

B. Cysts (Reference *Artemia* cysts), subcellular fractions

Subcellular fraction	Ratio
Total homogenate before centrifugation	0.88
Total yolk platelets	1.84
Fraction enriched in mitochondria	0.54
Post-mitochondrial supernatant	0.36
Flotating lipids	0.35
Lysed yolk platelets	
Insoluble fraction	2.40
Soluble fraction (containing lipovitellin)	1.02

C. Whole adults (San Francisco Bay strain fed on *Spirulina* algae)

	Ratio
Females, reproductively active	0.25–0.35
Males, reproductively active	<0.1
Preadults	<0.1

D. Organ distribution in reproductively active females

	Ratio	
	San Francisco Bay strain	Macau strain
Vitellogenic ovaries	1.82	2.15
Ripening eggs	1.86	1.87
Hemolymph	0.52	0.65
Gut	<0.1	<0.1
Carcass	<0.1	<0.1

E. Stereomutation mixtures

	Ratio
No formic acid present	0.41
Formic acid present	0.53

F. Extract of cysts (Macau, batch 870191) subjected to heating (55 °C)

Time (min)	Ratio
0	1.21
3	0.97
5	0.81
15	0.47
30	0.46

trans-canthaxanthin ratio remained constant until the point of hatching. After hatching, *cis*-canthaxanthins rapidly disappeared in the nauplii, as evidenced from the approximate 55% drop of the *cis/trans* ratio within the first 8 h of post-breaking naupliar development, and became undetectable in 2-day-old nauplii fed on dried algae (*Spirulina*). Sexual differentiation led to their reappearance in reproductively active females but not in males or in immature animals (preadults) from the same brood (Table IC). We examined the carotenoid distribution in female adults of two different strains by analyzing isolated body sections, including the ovaries, the ripening eggs, the gut, the hemolymph, and the remaining carcass. *cis*-Canthaxanthins were selectively localized in vitellogenic ovaries, the ripening eggs, and the hemolymph, while being virtually absent in the gut and the carcass (Table ID). In contrast, all-*trans*-canthaxanthin was present in all fractions, including the carcass. β-Carotene as well as some unidentified xanthophylls were found mainly in the gut. The selective localization of *cis*-canthaxanthins together with their virtual absence in males, developing nauplii, and preadults provides strong evidence for their nonartifactual character, particularly since all samples were analyzed under the same conditions. *trans* $\rightarrow$ *cis* isomerization, if occurring during sample pretreatment, would lead to an equilibrium *cis/trans* mixture (6). However, as shown in Table IE, the *cis/trans* ratios found in dehydrated cysts and especially in subcellular fractions largely exceed the equilibrium *cis/trans* ratio.

Further experimental evidence proving the nonartifactual nature of the *cis*-canthaxanthins in *Artemia* was obtained by supplementing cysts with all-*trans*-canthaxanthin before homogenization; no additional *cis*-canthaxanthin besides the amount naturally present was formed during analysis. Even storing the extracts for several hours in darkness at room temperature did not induce *trans* $\rightarrow$ *cis* conversion. On the contrary, the *cis/trans* ratio in extracts gradually decreased, indicating backward *cis* $\rightarrow$ *trans* isomerization. Heating the extract accelerated this process and rapidly led to a value close to the equilibrium ratio (Table IF). This clearly shows that *in vitro* stereomutation cannot account for the high *cis/trans* ratios observed in biological extracts.

DISCUSSION

The present report represents, to the best of our knowledge, the first example of the isolation of *cis*-carotenoids from animals, except for the eye-specific 11-*cis*-retinal, which is not a carotenoid strictly speaking. Only in plants (18, 19) and certain bacteria (20) have such isomers, often in trace amounts, been detected.

Any hypothesis regarding a possible function of *cis*-canthaxanthins in *Artemia* should focus on the fate of the carotenoid-protein complexes (1). Our observation that *cis*-canthaxanthins strongly bind to yolk platelets confirms earlier reports on the high affinity of egg yolk for carotenoids. Canthaxanthin-lipovitellin complexes have previously been demonstrated in several crustacea (21), including *Artemia* (22–24) and the closely related freshwater anostracan *Branchipus* (25, 26). On the basis of raman resonance and circular dichroism studies, Zagalsky *et al.* (24, 26) suggested a *cis*-conformation for part of the canthaxanthin bound to *Artemia* and *Branchipus* lipovitellin. Similar spectral evidence has indicated a *cis*-configuration for carotenoids in the reaction centers of certain photosynthetic bacteria (27–29). However, attempts to isolate *cis*-isomers from *Artemia* lipovitellin failed, even though a transient *cis*-peak could be observed in the absorption spectra of freshly prepared extracts (24).

Unlike all other carotenoids in *Artemia*, only *cis*-canthaxanthins occur at a specific site. All-*trans*-canthaxanthin is obviously nonspecifically distributed throughout the whole body, whereas the presence of β-carotene, its metabolic precursor (8–12), and xanthophylls in the gut merely reflects the algal diet.

The appearance of these specific pigments in the ovaries and eggs of reproductively active females suggests some kind of relation to reproduction and/or embryonic development. Current opinions on the role of carotenoids in Crustacea either advocate (4, 21) or completely deny (5) a precise function in this regard. The well established photoprotective capability of carotenoids (30) has been invoked to rationalize their concentration in the eggs of a copepod *i.e. Diaptomus*, the degree of pigmentation being dependent on sunlight intensity and predator pressure (31, 32). This organism was shown to undergo a seasonal pigmentation cycle, apparently correlating with reproductive periods. Unfortunately, both males and females exhibited this phenomenon. This is unlike our finding of a pigment appearing in close relation to reproduction but only in female animals and at a specific site. Our observation may thus provide a first clue to a previously unrecognized function for carotenoids in *Artemia* and possibly related Crustacea.

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